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Changes in bacterial flora during short-term storage of aquafeed and feed ingredients

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Abstract

Bacteriological changes in two shrimp feeds and protein rich ingredients on short-term storage for 60 days were studied. Feed I was a commercial shrimp 'grower' diet and Feed II, a cost effective diet for shrimp formulated at CMFRI, Cochin. The protein-rich feed ingredients used were fishmeal, groundnut oil cake and soybean flour. Sealed samples were stored under identical conditions at room temperature and were analyzed for bacterial population at 0, 30 and 60 days. The total plate count of feeds and feed ingredients were well within the reported non-degrading limit. Gram-positive cocci (42.8-73%) were dominant in all the five samples. During storage gram-positive cocci showed slight reduction, while, gram-negative rods showed marginal increase. Five hundred bacterial isolates derived from feed and feed ingredients were used for further characterization and identification. The major genera identified were *Micrococcus*, *Arthrobacter* and *Bacillus*. Besides, enumeration of indicator bacteria such as *Vibrio*, Faecal streptococci, *Escherichia coli* and *Staphylococcus aureus* was also done in the feeds and feed ingredients. Feed II was devoid of all the above indicator forms, except *Vibrio*, while, Feed I had all the above groups in varying numbers. Short-term storage (up to 60 d) did not have much effect on bacterial load in feeds and feed ingredients.

Key words : Aquaculture, Nutrition - Shelf - life, bacteria

Introduction

The success of shrimp farming depends upon scientific and sound management of all facets of aquaculture. Nutritional expenditure in shrimp culture amounts to about 40 to 60% of the operational costs. Parallel to intensification of shrimp farming industry, nutritionally balanced and high quality compounded diets were developed and commercialized (New, 1987,1990; Shigueno, 1984; Akiyama and Dominy, 1989; Paulraj, 1993; Tacon, 1993; Ali, 1997). Besides commercial aquafeeds, many farmers prepare feeds for captive consumption to reduce the cost of farming. Feed management is a sequential process comprising feed selection, handling and storage, feeding regimes and adjustments to feeding rates (New, 1990; De- la-Cruz *et al.*, 1989; Tacon, 1993; Akiyama and Chwang, 1995; Jory, 1995). Poor storage and handling of feeds result in product deterioration, reduced feed attractability and palatability, nutritional deficiencies and disease outbreaks (Jory, 1996). The ingredients used in feed for-

mulation partly determine the nature of its microflora (Trust and Wood, 1973). Commercial feeds do contain a mixed microflora including bacterial species causing potential harm to man and fish (Trust, 1975). The main objective of this work was to study the changes in bacterial profile during storage of selected feeds and feed ingredients for 60 days.

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Material and methods

The changes in bacterial flora during short-term storage (60 d) of two shrimp feeds and three protein-rich ingredients were studied. Feed I was a commercial grower shrimp feed collected from a local farmer at Cochin, Kerala. Feed II was a formulated diet from Central Marine Fisheries Research Institute. The feed ingredients viz. dry fish, groundnut oil cake (GNOC) and soybean flour were purchased from the local market. Dry fish and GNOC were dried in hot air oven at 60°C overnight and ground in a domestic grinder to make fine particles of ≤1mm size. One kilogram each of feed and feed ingredients was stored at room temperature in sealed polythene bags to avoid any aerial contamination. Sampling was done aseptically on days 0, 30 and 60.

The quantitative analysis of bacteria in feeds and feed ingredients included total plate count (TPC), *Staphylococcus aureus*, *Escherichia coli* and *Vibrio* count. For determining total plate counts, pour plate technique using Zobell marine agar 2216, was followed. For Faecal streptococci count pour plate technique on Kenner Faecal streptococci agar (KFA) was carried out. *Staphylococcus aureus* count was carried out by spread plate technique on Baired Parker agar (BPA). Black colonies with thin white margin and a zone of clearance around colonies were counted as *S. aureus*. *Escherichia coli* count was done on Tergitol-7 agar by following spread plate technique. Non-mucoid, non-raised lemon yellow colonies with or without occasional rets, brown at the center and yellow zones around were counted as *E. coli*. Streaking on Eosin methylene blue agar (EMB) was carried out for confirmation. Spread plate count technique on thiosulphate citrate bile salt sucrose agar (TCBS) was followed for *Vibrio*. Yellow flat smooth colonies with opaque centers and transparent peripheries, 2-3diameter. were taken as *Vibrio*. Bacterial count per gram of sample was calculated using the formula:

$$\frac{\text{Average count x dilution factor x 10}}{\text{Sample weight}}$$

For isolation and identification of colonies, about 20-30 cultures from each feed giving preferably a count of 30- 60 well separated colonies on Zobell marine agar were randomly selected and transferred into peptone water tubes. The broth cultures were purified on Zobell marine agar by streak dilution method. Identification of isolates up to the genus level was done following the scheme of Surendran and Gopakumar (1981).

Results

Quantitative variation of bacterial flora in feeds and feed ingredients: The bacterial loads determined in two shrimp feeds and three protein rich feed ingredients during 60 days storage are shown in Tables 1-5. In Feed I, the initial TPC was 2.75 x 10^3 cfu/g, which increased to 8.8 x 10^4 cfu/g on day 30 and then reduced to 2.73 x 10^4 cfu/g on day 60. The number of colony forming units (cfu) of *Vibrio* almost doubled from 3.0 to 6.4 x 10cfu/g on day 60. The Faecal streptococci count also showed a marginal increase. The *E. coli* count remained constant throughout i.e; 3 x 10 cfu/g. *Staphylococcus aureus* was recorded only on day 30 of storing (2.2 x 10^2cfu/g), while it was absent on 60th day of sampling (Table1).

In the Feed II, the TPC showed a decline on day 60. The total count of *Vibrio*, however increased from 2 x 10^1 to 4 x 10^1cfu/g on day 30 and remained the same on day 60. Faecal streptococci, *E. coli* and *S. aureus* were not recorded in this feed throughout the period of observation (Table 2).

In all the three feed ingredients there was variation in bacterial count during storage. The total plate count in fishmeal, which was 5.12-x10^3 cfu/g on day 0, showed a decline to 2.7 x 10^3 cfu/g on day 30. The counts of *Vibrio*, *E. coli* (2 x 10^1cfu/g), *S. aureus* (3 x 10^2cfu/g) and F. streptococci (6.5 x 10^2cfu/g and 5.1 x 10^2cfu/g) did not show any significant variation. F. streptococci and *S. aureus* were absent in fishmeal on day 60 (Table 3).

The total plate count of GNOC showed an increase from 3.3 x 10^3 cfu/g to 5.1 x 10^3 cfu/g on day 30 followed by a reduction to 2.5 x 10^3 cfu/g on day 60. *Vibrio* count remained almost the same (3 x 10^1cfu/g) with an increase on day 30 (1.2 x 10^2cfu/g). Faecal streptococci and *E. coli* showed little or no variation. *S aureus* though occurred on day 0 was not recorded during subsequent sampling (Table 4).

Table 1. *Variation of bacterial population in Feed I*

Days	TPC	*Vibrio* spp.	F.streptococci	*E. coli*	*S. aureus*
0	2.765 x10^3	3 x 10^1	3.6 x 10^1	3 x 10^1	Nil
30	8.8 x 10^4	5.3 x 10^1	2.9 x 10^1	3 x 10^1	2.2 x 10^2
60	2.73 x 10^4	6.4 x 10^1	4.5 x 10^1	3 x 10^1	Nil

Table 2. *Variation of bacterial population in Feed II*

Days	TPC	*Vibrio* spp.	*F. streptococci*	*E. coli*	*S. aureus*
0	5.75 x 10^3	2 x 10^1	Nil	Nil	Nil
30	5.5 x 10^4	4 x 10^1	Nil	Nil	Nil
60	3.65 x 10^4	4 x 10^1	Nil	Nil	Nil

Table 3. *Variation of bacterial population in Fishmeal*

Days	TPC	*Vibrio* spp.	F. streptococci	*E. coli*	*S. aureus*
0	5.12×10^3	2×10^1	6.5×10^2	2×10^1	Nil
30	3.87×10^3	3×10^1	5.1×10^2	2×10^1	2.2×10^2
60	2.7×10^3	2×10^1	Nil	2×10^1	Nil

Table 4. *Variation of bacterial population in groundnut oil cake*

Days	TPC	*Vibrio* spp.	F. streptococci	*E. coli*	*S. aureus*
0	3.3×10^3	3×10^1	6×10^1	3×10^1	2.3×10^2
30	5.1×10^3	1.2×10^2	5×10^1	3×10^1	Nil
60	2.51×10^3	3×10^1	4.9×10^1	3×10^1	Nil

Table 5. *Variation of bacterial population in soybean flour*

Days	TPC	*Vibrio* spp.	F. streptococci	*E. coli*	*S. aureus*
0	3.15×10^3	2×10^1	Nil	Nil	3.5×10^2
30	2.075×10^3	1.0×10^2	Nil	Nil	3.1×10^2
60	2.75×10^3	2×10^1	Nil	Nil	Nil

Total plate count of soybean flour showed a decline from 3.15×10^3 cfu/g to 2.75×10^3cfu/g on day 60. *Vibrio* did not show much quantitative variation and remained at 2×10^1cfu/g. Faceal streptococci and *E. coli* were absent in the soybean flour.

Qualitative analysis of bacterial genera in feeds and feed ingredients: For qualitative study 500 isolates from feeds and feed ingredients were maintained in Zobell marine agar slants. *Vibrio* from TCBS media were gram-negative rods, oxidase and catalase positive with round, entire, glossy and yellow or green colonies. Faceal streptococci plated on KFA medium were gram positive, facultatively anaerobic, non-motile, catalase and oxidase negative. *E. coli* plated on Tergitol-7 agar were gram-negative, motile, and oxidative. The positive indole test was used as confirmative test. *S. aureus* plated on BPA medium was coccoid form, gram positive, facultatively anaerobic, and non-motile and catalase positive.

The percentage composition of different bacterial genera in the feeds as well as feed ingredients is shown in Figures 1 to 5. The commonest genus was *Micro-*

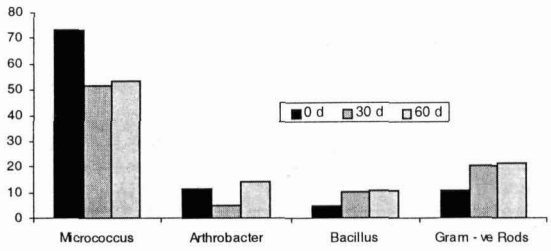

Fig. 1. *Percentage occurrence of different bacterial genera in Feed I*

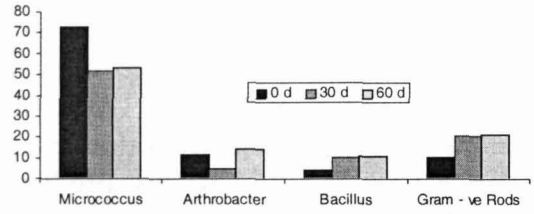

Fig. 2. *Percentage occurrence of different bacterial genera in Feed II*

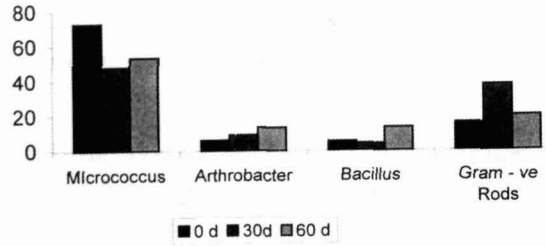

Fig. 3. *Percentage occurrence of different bacterial genera in Fishmeal*

coccus in both the feeds and feed ingredients. Other genera were *Arthrobacter* and *Bacillus*. The occurrence of these genera did not show much variation on storage. Gram-negative rods increased from 13.6% to 23.8% on day 60 in Feed I. In Feed II, *Micrococcus* got reduced from 73% to 53.2% on day 60; while *Arthrobacter* (11.5% to 14.7%), *Bacillus* (4.6%- 10.7%) and gram-negative rods (10.6 - 10.7%) showed increase during storage.

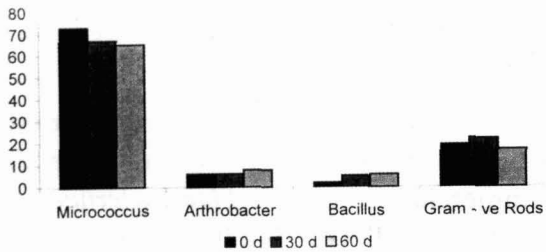

Fig. 4. *Percentage composition of different bacterial genera in groundnut oil cake*

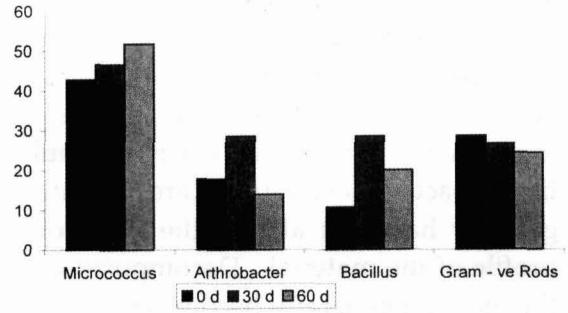

Fig. 5 *Percentage occurrence of different bacterial genera in Soybean flour*

In fishmeal, *Micrococcus* showed a decrease from 73% to 65.1% on day 60. *Arthrobacter* (6 - 7.7%) and *Bacillus* (2.0 - 5.7%) showed slight increase, whereas, gram- negative rods showed slight increase on day 30 (22%) and then decreased on day 60 (19.0 - 17%). In GNOC, *Micrococcus* showed a decrease during storage (73% to 53.3%), while, *Arthrobacter*, *Bacillus* and gram-negative rods; 6-13.3%, 5.3- 13.3%, and15.7 to 20% respectively, showed marked increase on day 60.

In soybean flour alone *Micrococcus* constituted less than 50% (42- 48%) and showed marginal increase on storage (42.8 - 51.7%) while, *Arthrobacter* formed 17.8 to 28.5% on day 30 and then reduced to 23.9% on day 60. Gram-negative rods showed a gradual reduction during storage (28.5 to 24.3%). *Bacillus* also showed an increase on day 30 (28.5%) and then reduced to 20% on day 60.

Discussion

The results of bacterial analysis suggest that the selected shrimp feeds and feed ingredients are susceptible for bacterial growth and thus are not sterile. Because

feed is a source of nutrients required for growth, the presence of bacterial inoculum promotes their growth and multiplication. Feeds are considered unsatisfactory when they contain a large population of bacteria, even if they are not pathogenic or have not altered the nutritional profile of the material. Decomposition of the material generally becomes evident in food containing 10^6 to 10^8 organisms/g (Thatcher and Clarke, 1968). Although the total plate counts (TPC) in both the feeds as well as feed ingredients examined in the present study were well within this range during 60 days storage, the ability of the bacteria to produce extracellular proteolytic, lipolytic and amylolytic enzymes would favour spoilage if any change in physico-chemical parameters like moisture, temperature, *p*H, *etc.* occurred during storage. Many workers have reported higher ranges of bacterial count in different types of fish diets (Sera and Kimata, 1971; Trust, 1971 and Trust and Wood, 1973).

Gram-positive cocci were the dominant group in both feeds as well as feed ingredients in the present study, which is in conformity with the observations of. Muroga *et al.*(1987). Other dominant genera were *Arthrobacter* and *Bacillus*. Factors like type of microflora present in the ingredients used in feed preparation, type of heat treatment that the feed has undergone and the moisture content would have influenced the final bacterial load of diet (Trust and Wood, 1973; Blank *et al.*, 1996). Christian (1980) had observed a shift from gram-negative to gram-positive flora in proteinaceous foods when the water activity dropped to 0.98 to 0.96. As a result of this, the major bacterial flora of such feed could be gram-positive. According to Surendran and Gopakumar (1981), the gram-positive cocci, usually found in association with fish and fishery products or aquatic environments are either of three genera, viz. *Staphylococcus*, *Streptococcus* or *Micrococcus*. Since *Staphylococcus* and *Streptococci* were absent in Feed II, it can be inferred that gram-positive cocci observed in Feed II belonged to the genus *Micrococcus*.

Sugita *et al.* (1986, 1987) have studied the microflora of pellet diets and found them to be composed of *Bacillus* (4.8×10^3/g) and *Clostridium* (6×10^2/g). The only bacterial genus reported from gray mullet diet was *Bacillus* (Hamid *et al.*, 1978). In the present study *Bacillus* constituted 4.5-10% in feeds and 2.0-20% in feed ingredients. The gradual increase observed in gram-negative bacteria in both the feeds on storage may be attributed to its dominance over gram-positive genera under favourable conditions, which on prolonged storage may altogether replace gram- positive ones. A gradual increase of *Arthrobacter* was observed in Feed I, Feed II, fishmeal, and GNOC. In soybean flour, *Arthrobacter* and *Bacillus* were maximum on day 30.

Ringo and Strom (1994) have reported predominance of *Enterobacteriaceae* in commercial feeds and *Flavobacterium* in capelin roe diet. It is also a well-established fact that feed consumed by cultured aquatic organisms does have a

definite influence on the gut microbial flora (Del-Rio-Rodriguez *et al.*, 1997). Ogbondeminu *et al.* (1991) have reported that 80% of the feed microflora were identified in the water, and of the bacterial population in feed 25% was *Pseudomonas*, 10% *Staphylococcus*, 12% *Streptococcus*, 15% *Micrococcus* and *Aeromonas* 8%. The fact that *Staphylococcus*, *Streptococcus faecalis*, *etc.*, are facultative pathogens or agents of food poisoning is of importance.

Although the presence of these bacteria is not often associated with fish diseases or enteric diseases in man, the health implications, on the introduction of these organisms into natural water via the unconsumed feed and faeces in aquaculture waste water cannot be ignored. *Vibrio*, Faecal streptococci, *E. coli* and *S. aureus*, *etc.*, are indicators of contamination as well as potential pathogens. The feed containing these bacteria would form an inoculum in culture systems either through the uneaten feed or through faeces. The presence of indicator or pathogens or any other bacteria beyond limit is therefore dangerous to the system (Trust, 1971). In the present study *Vibrio* counts in Feed I increased during storage, while in Feed II it gradually increased on day 30 and remained stable till day 60. For feed ingredients also slight variation in *Vibrio* counts was observed with a reduction on day 30 and increase on day 60. Reilly and Twiddy (1992) in their attempt to identify the source of *V. cholerae* in shrimp feeds have concluded that commercial shrimp feed was a potential source of the pathogen. From the present observations it can be assumed that the source of *Vibrio* may be the feed ingredients especially fishmeal and groundnut oil cake. *V. parahaemolyticus*, another potentially pathogenic species reported in cultured shrimp and seafood, is dangerous for human consumption (Karunasagar *et al.*, 1990). Muroga *et al.* (1987) have reported 10% *Vibrio* from *Artemia* and rotifers.

Faceal streptococci showed slight increase in Feed I on day 60, while it was absent in Feed II, which was formulated at Central Marine Fisheries Research Institute. In fishmeal and GNOC, it got reduced on day 60 whereas it was absent probably due to the fact that it was of food grade.

E. coli count in Feed I, fishmeal and GNOC remained stable throughout the 60 day storage period indicating that storage did not have any effect on its population in aquafeeds and feed ingredients. Trust and Money (1972) reported that *Enterobacteriaceae* in feeds could pose potential contamination with enteric pathogens. Feed II and soybean flour did not harbor *E. coli* in the present study.

In Feed I and fishmeal, *S. aureus* appeared on day 30 and was absent on the day 60, whereas it was absent throughout the storage period in Feed II. In GNOC and soybean initially it was present, which then disappeared on storage probably due to the dominance of other groups or possibly due to the inhibition of the strain during storage as reported for *Salmonella enteritis* in fishmeal during storage (Pelagic *et al.*, 1998). The processing method

employed during feed preparation especially heat treatment seems to destroy the bacteria which may be the reason for the absence of Faceal streptococci, *E. coli* and *S. aureus* in feed II, which was formulated at the Nutrition Laboratory of CMFRI.

The presence of indicator organisms found in the feeds as well as ingredients requires adequate consideration. For human food, the permitted total aerobic plate count is ≤10,000/g, total coliform <10/g, faecal coliforms nil and Faecal streptococci <20/g (Trust, 1971). Although these requirements may be too rigorous for aquafeeds, the presence of indicators and pathogens are unacceptable if the cultured organisms fed on the rations harboring these microorganisms are for human consumption. Therefore, restriction should be imposed on shrimp feed also, because fish have been shown to be vectors of disease causing bacteria in human beings (Jansen, 1970). There is paucity of published information pertaining to changes in bacterial flora of feed ingredients on storage. Therefore, the present observations form a baseline, but clearly reveal the importance of using good quality feed ingredients for aquafeed manufacturing.

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